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# Determination of the Optimal Ammonium Sulfate Concentration for the Fractionation of Rabbit, Sheep, Horse, and Goat Antisera

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Various ammonium sulfate concentrations and reaction conditions were employed in the fractionation of sera from rabbits, sheep, horses, and goats. Precipitates and supernatant fluids were analyzed by electrophoresis to study the effects of the controlled variables. At room temperature, the third precipitate in 35% saturated $(NH_4)_2SO_4$ was the best fraction from both rabbit and sheep sera; 80 to 90% of the gamma globulins were recovered. The second and third precipitates of horse sera proteins in 30% saturated $(NH_4)_2SO_4$ were both satisfactory, but only 44% of the gamma globulin was recovered after three precipitations. Goat sera yielded a very satisfactory fraction; 80% of the gamma globulin was recovered after two precipitations—the first in 30% and the second in 45% saturated $(NH_4)_2SO_4$. The composition of these fractions was not influenced by the *p*H of the sulfate solutions (*p*H 5.8 and 7.2), by a range of normal room temperatures (20 to 30 C), or by diluting the sera before fractionation. Crude globulins and fluorescein isothiocyanate-labeled globulins were successfully refractionated by one precipitation in the optimal sulfate concentration for the appropriate animal species. The refractionated products contained considerably less beta and alpha globulins than did the original crude fractions and little or no albumin.

The most frequently used method for fractionating antisera is precipitation in the cold with half-saturated ammonium sulfate. The procedure is simple and inexpensive, and the resulting crude antibody fractions from the antisera of a number of animal species have been successfully used in many areas of immunology. In this laboratory these crude fractions have been conjugated with fluorescein isothiocyanate (FITC) for use as fluorescent-antibody (FA) reagents. During a series of studies designed to improve FA methodology, these crude fractions were analyzed by cellulose acetate strip electrophoresis. The results revealed that most of the fractions contained less than 50% gamma globulin and that many of them contained undesirable amounts of albumin. A survey of commercial antibacterial FA reagents also had shown a need for improved fractionation procedures (5). This need for improved fractionation coupled with the need to retain the simple and inexpensive aspects of the $(NH_4)_2SO_4$ procedure led to this systematic study of various concentrations of the salt for serum fractionation. (Presented in part at the Annual Meeting of the American Society for Microbiology, Minneapolis, Minn., 3 May 1971.).

## MATERIALS AND METHODS

**Antisera.** The antisera used in these studies were produced in rabbits, sheep, horses, and goats against a variety of bacterial antigens including *Bacillus anthracis*, *Bordetella bronchiseptica*, *Bordetella pertussis*, *Escherichia coli*, *Pseudomonas pseudomallei*, *Salmonella*, *Shigella dysenteriae*, *Shigella sonnei*, and *Yersinia pestis*. Some normal sera were also used.

**Ammonium sulfate.** A stock solution of saturated ammonium sulfate (SAS) was prepared and stored at room temperature (approximately 25 C). Working solutions of 50, 60, 70, 80, and 90% SAS were prepared (v/v) fresh as needed from the stock saturated solution. Equal volumes of these solutions and various antisera resulted in reaction mixtures of 25, 30, 35, 40, 45, and 50% SAS (6).

**Fractionation.** The following procedure for frac-

tionation was used. A volume of serum was gently stirred while an equal volume of an ammonium sulfate solution was slowly added and mixed well. The reaction mixture was set aside at room temperature for 4 hr and then centrifuged to pack the precipitated protein. The supernatant fluid was removed and stored for later analysis. The precipitate was resuspended and dissolved in distilled water to a final volume equal to the original volume of serum. For a second precipitation, the dissolved protein was gently stirred while an equal volume of an ammonium sulfate solution was slowly added. The mixture was immediately centrifuged to pack the formed precipitate, and the supernatant fluid was discarded. The precipitate was dissolved and brought to volume as before. A third precipitation was handled in the same manner.

All fractions were dialyzed against frequent changes (3) of *p*H 8, 0.85% NaCl solution until sulfate was no longer detected in the dialysate (7). The saline was brought to *p*H 8 with a few drops of 10% NaOH. A small volume of saturated barium chloride solution was added to an equal volume of well-mixed saline dialysate to check for the presence of sulfate. If no cloudiness resulted, the dialyzed fraction was considered substantially free of sulfate.

**Protein.** Protein concentrations were measured by the Biuret method (2) with a Beckman DB spectrophotometer. Protein compositions were determined by cellulose acetate strip electrophoresis (CASE) with the Beckman Microzone (1) equipment and procedure with a slight modification. After the membranes were stained with Ponceau S, they were rinsed in 5% acetic acid until the background was white; then they were gently blotted to remove excess moisture and placed between dry blotters on a flat surface beneath a glass thin-layer chromatography plate to dry. After drying, the uncleared membranes were read on a Beckman Densitometer, model R-110, at the recommended settings.

**Electrophoresis interpretation.** The integrated densitometer tracings of the proteins subjected to electrophoresis were interpreted by the following method (Fig. 1). (i) Each of these proteins, when isolated and studied in the pure state, has a peak of Gaussian configuration. Use Gaussian projections to extrapolate to the base line the right side of the gamma peak and the left side of the albumin peak. (ii) Drop perpendiculars through these projections so that an area "A" between the original tracing and the projection is equal to an area "B" inside the projection and on the opposite side of the perpendicular. Extend these perpendiculars down through the integrator trace. (iii) The integrated tracing of the proteins subjected to electrophoresis has been partitioned into three regions—gamma globulin, beta and alpha globulins, and albumin. To calculate the area in each region, add the individual counts registered for each region and obtain the relative proportion of each.

**Fluorescein and fluorescein to protein ratios.** Fluorescein isothiocyanate was determined as protein-bound FITC by absorbance at λ max (near 495 nm) in 0.1 N NaOH, and related to a pure fluorescein diacetate reference standard (9). The fluorescein to protein (F/P) ratio was calculated from the FITC and protein measurements and expressed as micrograms of protein-bound FITC per milligram of protein.

**Specific antibody titration.** The specific staining titers of all conjugates were determined by routine FA staining procedures (10, 11). The highest dilution, which gave a 4+ staining of the bacterial cells, was recorded as the specific titer.

## RESULTS

**Preliminary studies.** Many of these studies were done with pools of animal sera rather than individual sera. Once a procedure was worked out it was applied to other pooled sera as well

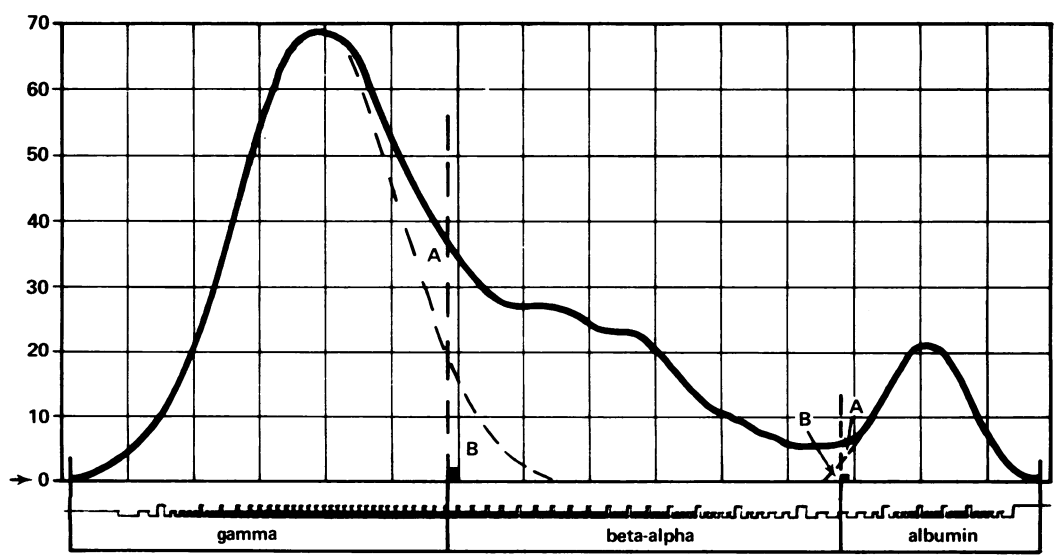



FIG. 1. *Method used to interpret electrophoresis tracings.*

as individual antisera. The electrophoresis profiles of rabbit, sheep, horse, and goat sera pools are shown in Fig. 2. They are easily distinguished from each other, and each is very characteristic of its species. The rabbit profile is the least distinctive and is quite similar to the familiar human serum profile. The sheep gamma globulin resolves as two separate peaks. Horse serum profiles exhibit two strong peaks in the alpha globulin region. In goat serum, gamma is the dominant globulin. These features remain constant within the species, and the differences in individual animals are minor except for fluctuations in the gamma globulin concentrations, which reflect immunological status.

The CASE profiles of serum fractions obtained after three precipitations of these four sera in 50% SAS are shown in Fig. 3. The numerical data extracted from Fig. 2 and 3 are presented in Table 1. These crude fractions contained only 23 to 48% gamma globulin and as much as 9 to 21% albumin. All of the gamma globulins present in the original sera were recovered, but many other proteins were also precipitated.

**Rabbit studies.** The CASE profiles of the first, second, and third precipitations of rabbit *E. coli* antisera in 50, 45, 40, and 35% SAS are shown in Fig. 4. These tracings show that, as the concentration of ammonium sulfate was decreased, the gamma globulin percentage in the

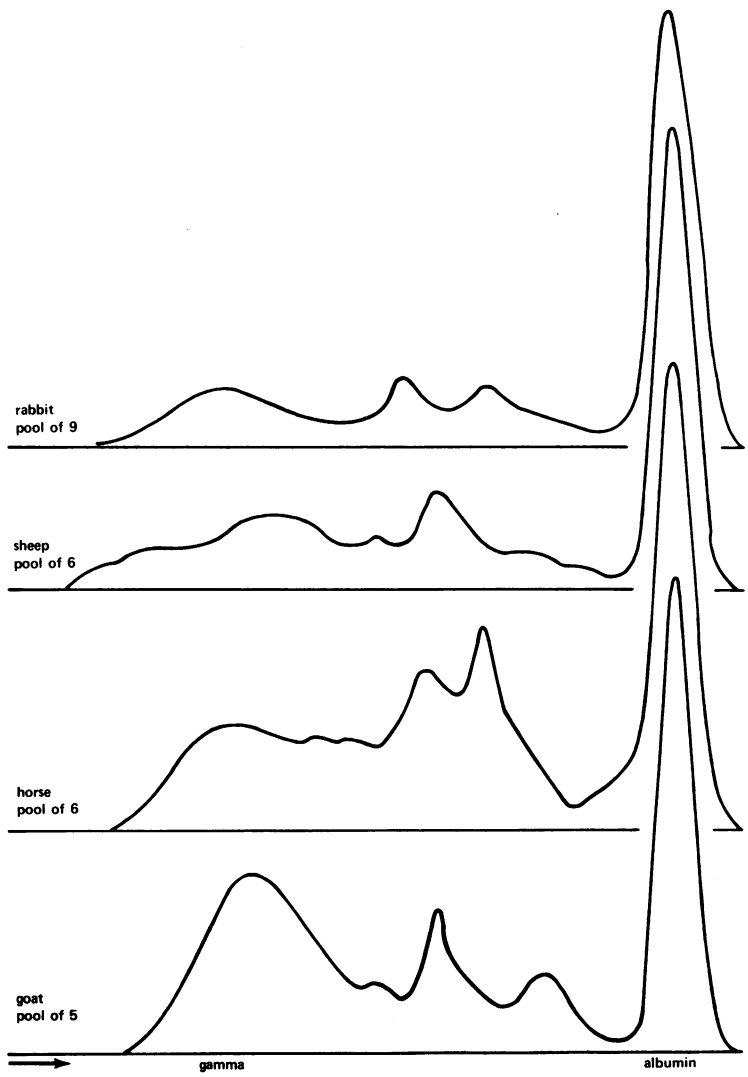



FIG. 2. *Electrophoretic profiles of pooled animal sera.*

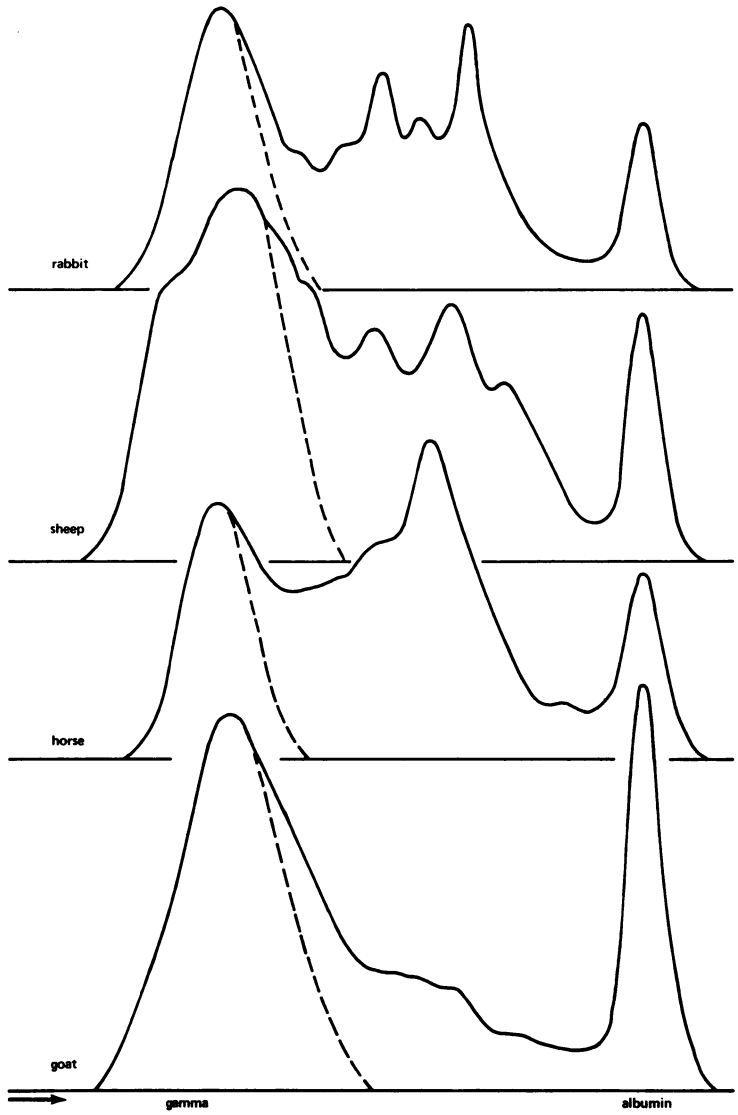



FIG. 3. *Electrophoretic profiles of animal serum fractions after three precipitations in 50% saturated* $(NH_4)_2SO_4$

precipitates was increased. Consecutive precipitations with a given concentration of ammonium sulfate did not change the general ratio of the amount of gamma globulin to the amount of beta and alpha globulins, but they did reduce the percentage of albumin in the precipitate. The third precipitation in 35% SAS gave a fraction which was 65% gamma globulin and only 1% albumin. Very little precipitate formed in 30% SAS, and its composition was not desirable. No precipitation occurred in 25% SAS. Seven other bacterial antisera of rabbit origin were fractionated by three consecutive precipitations in 35% SAS. The data from CASE of all eight antisera and their sulfate fractions are given in Table 2. Gamma globulin in the original antisera ranged from 5 to 21%, and the fractions contained from 54 to 68% gamma globulin with a maximum of 2% albumin.

**Sheep studies.** The CASE profiles of the first supernatant fractions and the third consecutive precipitates of sheep serum in 45, 40, 35, and 30% SAS are shown in Fig. 5. With sheep serum as with rabbit serum, decreasing the sulfate concentration resulted in an in-

creased percentage of gamma globulin in the precipitate, and repeated precipitations in a given SAS concentration yielded reduced percentages of albumin. The third precipitation in 35% SAS gave a fraction containing 68% gamma globulin and less than 1% albumin. With this amount of sulfate, a small amount of gamma globulin was lost in the first supernatant fraction, but considerably more was lost in the first supernatant fraction obtained with 30% SAS without improvement in the composition of the third precipitate.

**Horse studies.** CASE profiles of the first supernatant fractions and third precipitates of horse serum in 45, 40, 35, and 30% SAS are shown in Fig. 6. Decreasing the sulfate concen-

TABLE 1. *Proteins in four animal serum pools and their 50% saturated $(NH_4)_2SO_4$ fractions*

| Serum pool | Composition by CASE[a] | | |
|---|---|---|---|
| | % gamma | % beta-alpha | % albumin |
| Rabbit: Original | 17 | 25 | 58 |
| Fraction[b] | 33 | 58 | 9 |
| Sheep: Original | 26 | 32 | 42 |
| Fraction | 44 | 47 | 9 |
| Horse: Original | 20 | 46 | 34 |
| Fraction | 23 | 65 | 12 |
| Goat: Original | 30 | 36 | 34 |
| Fraction | 48 | 31 | 21 |

[a] Cellulose acetate strip electrophoresis.
[b] Crude fraction after three precipitations in 50% saturated $(NH_4)_2SO_4$.

TABLE 2. *Electrophoretic results from eight rabbit bacterial antisera and their fractions obtained with optimal[a] $(NH_4)_2SO_4$ concentrations*

| Antisera | | Composition by CASE[b] | | |
|---|---|---|---|---|
| | | % gamma | % beta-alpha | % albumin |
| *P. pseudomallei*: | Original | 5 | 26 | 69 |
| | Fraction[c] | 54 | 44 | 2 |
| *E. coli*: | Original | 7 | 27 | 66 |
| | Fraction | 65 | 34 | 1 |
| *Y. pestis*: | Original | 8 | 37 | 55 |
| | Fraction | 61 | 38 | 1 |
| *S. dysenteriae*: | Original | 10 | 34 | 56 |
| | Fraction | 68 | 32 | 0 |
| *B. bronchiseptica*: | Original | 12 | 33 | 55 |
| | Fraction | 55 | 45 | 0 |
| *B. pertussis*: | Original | 14 | 34 | 52 |
| | Fraction | 64 | 35 | 1 |
| *B. anthracis*: | Original | 17 | 38 | 45 |
| | Fraction | 66 | 34 | 0 |
| *Salmonella*: | Original | 21 | 36 | 43 |
| | Fraction | 59 | 40 | 1 |

[a] Determined in the present study.
[b] Cellulose acetate strip electrophoresis.
[c] Antiserum fraction after three precipitations in 35% saturated $(NH_4)_2SO_4$.

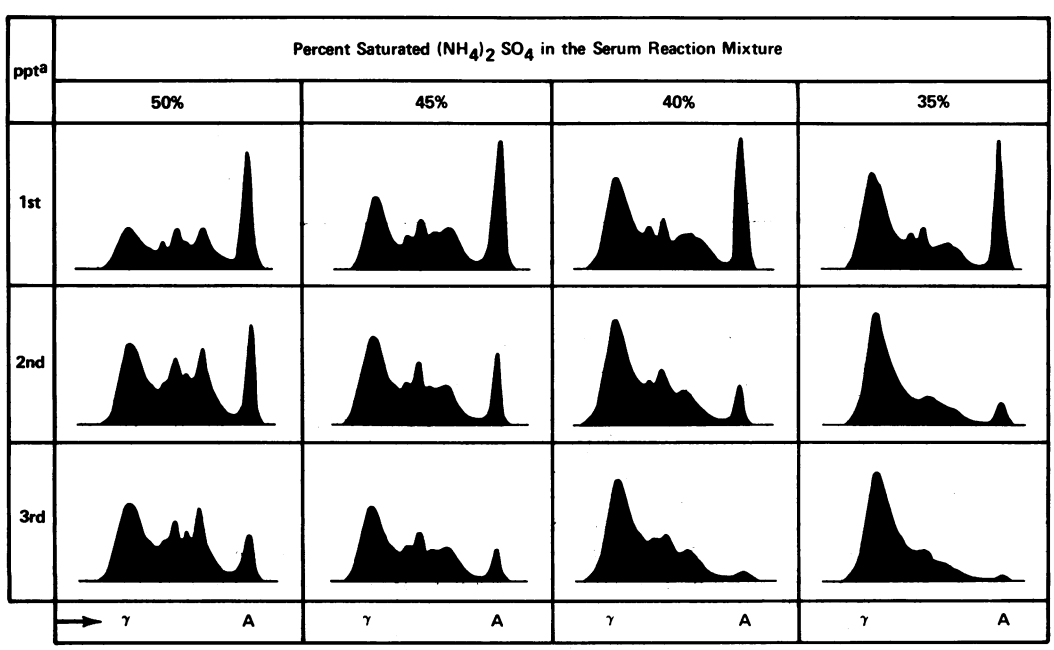



FIG. 4. *Electrophoretic profiles of $(NH_4)_2SO_4$ fractions of rabbit antiserum for E. coli. [a] Precipitation.*

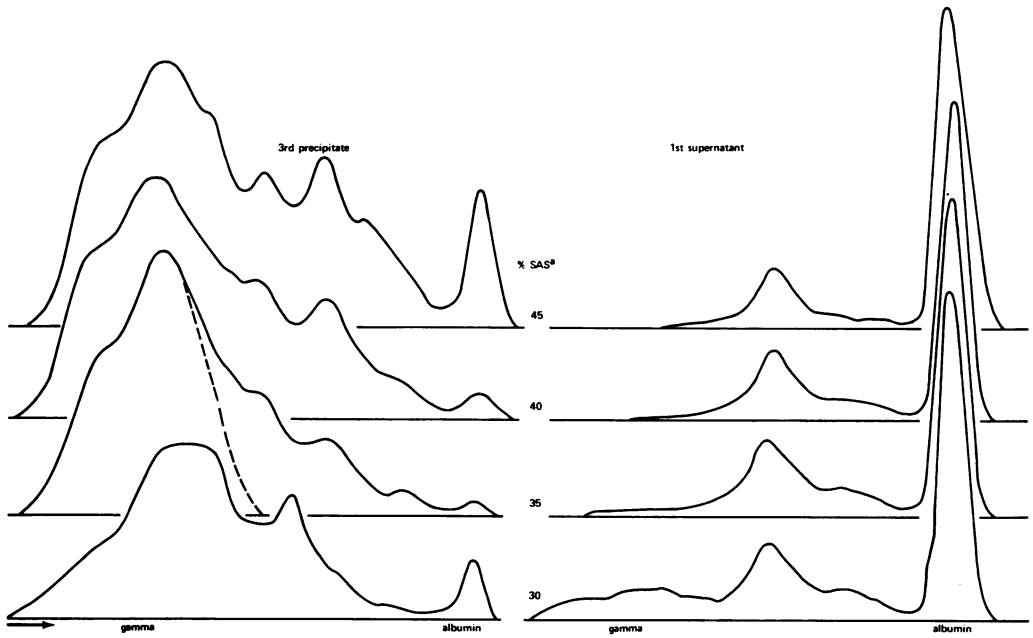



FIG. 5. *Electrophoretic profiles of sheep serum fractions obtained with* $(NH_4)_2SO_4$. [a] *Saturated ammonium sulfate.*

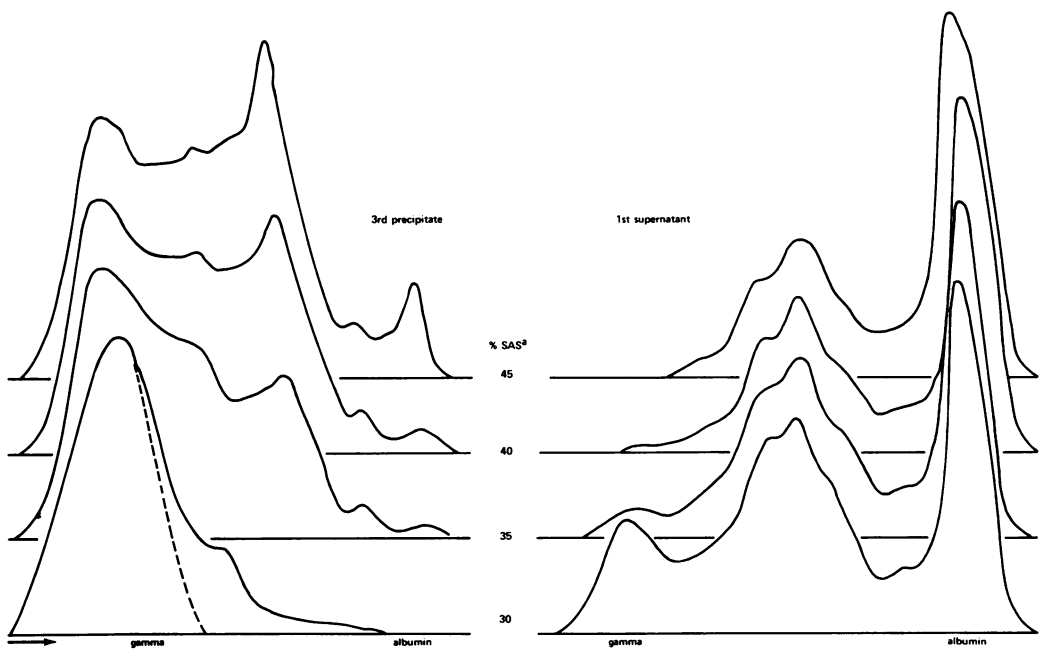



FIG. 6. *Electrophoretic profiles of horse serum fractions obtained with* $(NH_4)_2SO_4$. [a] *Saturated ammonium sulfate.*

tration had the same general effect on horse serum as that described for rabbit and sheep sera. At 35% SAS some gamma globulin remained in the supernatant fraction, but the third precipitate still contained a high percentage of beta and alpha globulins. Lowering the concentration of SAS to 30% left a large amount of gamma globulin in the first supernatant fraction but gave a third precipitate which was 83% gamma globulin with no albumin.

**Goat studies.** CASE profiles of goat *E. coli* antiserum fractions are shown in Fig. 7. Decreasing the sulfate concentration caused a greater change in the composition of the first precipitate of goat antiserum than it had with any of the other three animal sera. The first precipitate in 30% SAS contained 71% gamma globulin and only 6% albumin. No precipitate formed when a second sample of 60% SAS was used. When a 45% concentration of SAS was used to refractionate the 30% SAS precipitate, a fraction containing 81% gamma globulin and only 1% albumin was obtained.

**Recommended optimals.** The CASE profiles of the improved fractions from rabbit, sheep, horse, and goat sera, obtained by using the optimal ammonium sulfate concentrations for precipitation, are shown in Fig. 8. Rabbit and sheep sera were handled in the same way with three consecutive precipitations in 35% SAS. Horse serum required three precipitations in 30% SAS to effectively remove its high percentage of beta and alpha globulins. Goat serum required precipitation in 30% SAS followed by precipitation in 45% SAS. Under these conditions the percentage of gamma globulin recovered from the sera of rabbits, sheep, and goats was above 80%, and from that of horses, slightly less than 50% (Table 3).

**Refractionation.** Crude globulin fractions of rabbit antisera which had been prepared several years earlier by three precipitations in 50% SAS were subjected to a single precipitation in 35% SAS. The improvement was demonstrated by CASE profiles (Fig. 9). Several FA conjugates for *Salmonella* and *Shigella* detection have also been improved by a single precipitation in the optimal ammonium sulfate concentration appropriate for the animal species involved. The data for one of the *Shigella* conjugates are shown in Fig. 10. Refractionated conjugates were dialyzed in *p*H 7.6 phosphate-buffered saline (PBS) for sulfate removal. Since PBS reacts with saturated $BaCl_2$ to form a precipitate, $BaCl_2$ could not be used to check for the presence of sulfate; therefore, the dialysis time was extended to insure complete removal of the sulfate. Both globulins and conjugates were composed of considerably less albumin and beta-alpha globulins after one optimal precipitation. The *Shigella* conjugate lost 30% of its total protein, but only 7% of its gamma globulin. Although its FITC concentration was reduced 55% and the F/P ratio 36%, the specific antibody titer did not change.

**Effect of *p*H.** The unadjusted *p*H of all of the ammonium sulfate solutions as measured on the *p*H meter was 5.8. The *p*H of all serum-sulfate reaction mixtures ranged from 7.2 to 7.6, depending upon the *p*H of the original serum. Solutions of 90, 80, and 70% SAS were raised to *p*H 7.2 with 10% NaOH. Rabbit *B. bronchiseptica* antiserum was then fractionated in 45, 40, and 35% SAS with both

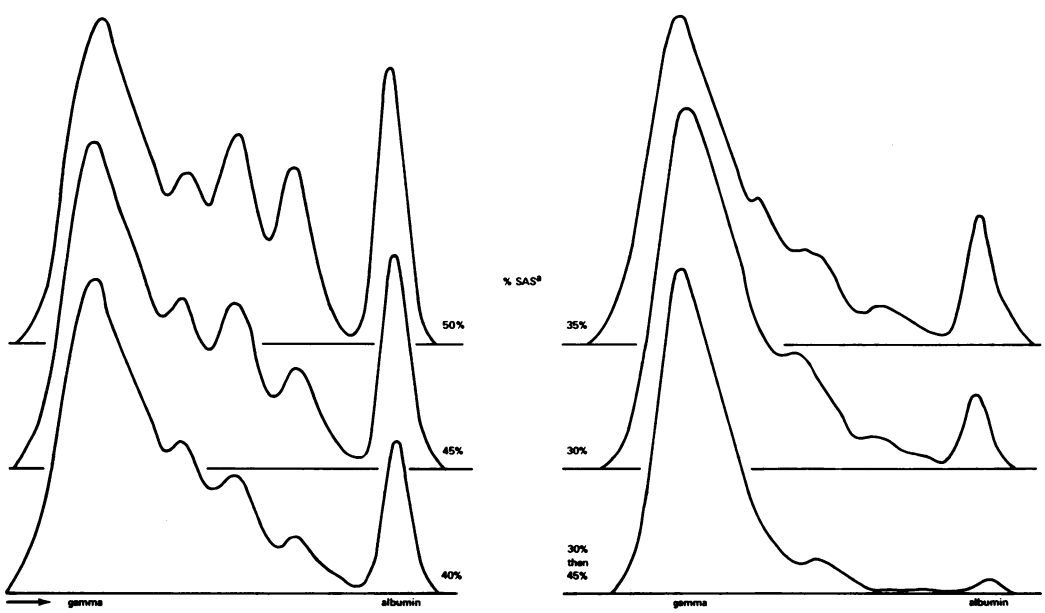



FIG. 7. *Electrophoretic profiles of goat E. coli antiserum precipitates obtained after one fractionation with various concentrations of* $(NH_4)_2SO_4$. [a] Saturated ammonium sulfate.

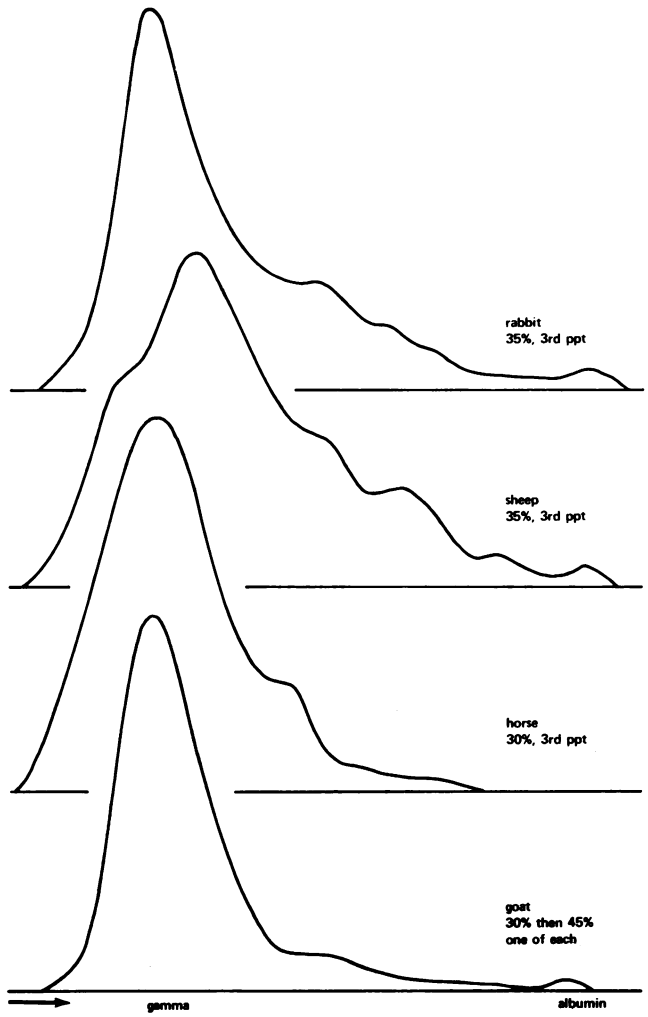



FIG. 8. *Electrophoretic profiles of antisera fractions obtained with optimal percent saturated solutions of* $(NH_4)_2SO_4$. *ppt, Precipitate.*

TABLE 3. *Recovery of gamma globulin from antisera after fractionation with optimal*[a] $(NH_4)_2SO_4$ *concentrations*

| Antisera | | % SAS[b] | No. ppts[c] | Protein (mg/ml) | % gamma CASE[d] | Gamma (mg/ml) | % gamma recovery |
|---|---|---|---|---|---|---|---|
| Rabbit: | Original | NA[e] | NA | 67 | 17 | 11 | NA |
| | Fraction | 35 | 3 | 15 | 66 | 10 | 91 |
| Sheep: | Original | NA | NA | 74 | 26 | 19 | NA |
| | Fraction | 35 | 3 | 24 | 68 | 16 | 84 |
| Horse: | Original | NA | NA | 81 | 20 | 16 | NA |
| | Fraction | 30 | 3 | 8.4 | 83 | 7 | 44 |
| Goat: | Original | NA | NA | 60 | 30 | 18 | NA |
| | Fraction | 30,45 | 1,1 | 18 | 81 | 15 | 83 |

[a] Determined in the present study.
[b] Saturated $(NH_4)_2SO_4$.
[c] Precipitations.
[d] Cellulose acetate strip electrophoresis.
[e] Not applicable.

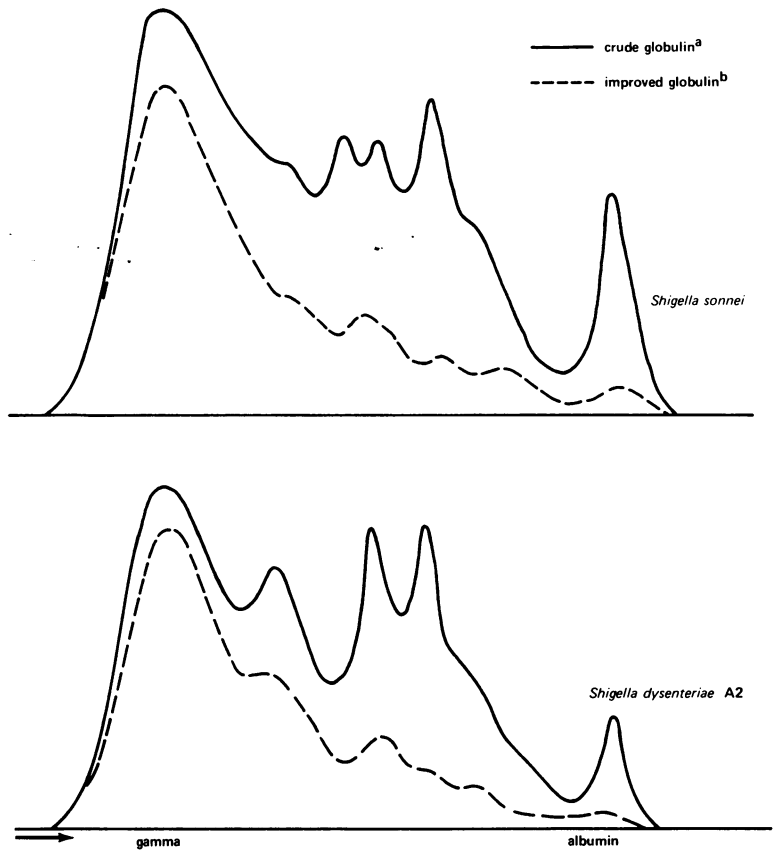
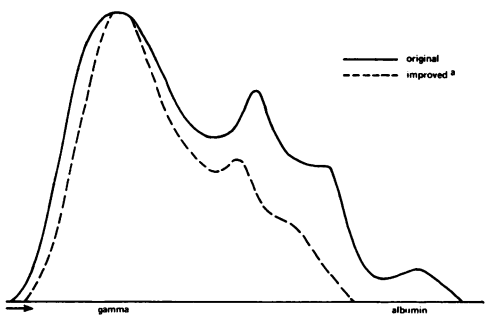



FIG. 9. *Improvement in crude globulins from Shigella rabbit antisera after one precipitation with the optimal $(NH_4)_2SO_4$ concentration. The crude globulin (a) was obtained after three precipitations in 50% saturated $(NH_4)_2SO_4$. The improved globulin (b) was obtained after one additional precipitation of the crude globulin, using 35% saturated $(NH_4)_2SO_4$.*




| conjugates | protein mg/ml | % gamma CASE[b] | gamma mg/ml | F/P[c] ratio | FITC[d] µg/ml |
|---|---|---|---|---|---|
| original | 10.8 | 49 | 5.3 | 14 | 149 |
| improved | 7.6 | 65 | 4.9 | 9 | 67.5 |
| % change | −30 | +16 | −7 | −36 | −55 |

FIG. 10. *Improvement in rabbit Shigella dysenteriae (A1) conjugate after one precipitation with the optimal $(NH_4)_2SO_4$ concentration. [a] Original conjugate was fractionated by one precipitation in 35% saturated $(NH_4)_2SO_4$. [b] Cellulose acetate strip electorphoresis. [c] Fluorescein to protein ratio. [d] Fluorescein isothiocyanate.*

the adjusted *p*H 7.2 reagents and unadjusted *p*H 5.8 reagents. After three consecutive precipitations under the six different conditions, the three fractions from the *p*H 7.2 sulfate solutions were practically identical to those obtained with the *p*H 5.8 reagents (Table 4).

**Effect of dilution.** Some rabbit *B. bronchiseptica* antiserum was also used to study the effect of dilution of serum prior to fractionation. Equal volumes of the original antiserum and of a 1:2 and 1:10 dilutions were each precipitated three times in 40% SAS. The final fractions had practically identical CASE profiles (Table 5).

**Effect of temperature.** To examine the possible influence of room temperature variations, we prepared and stored saturated solutions of ammonium sulfate at 20, 25, and 30 C. At each temperature, 70% SAS was prepared and used to fractionate rabbit *B. bronchiseptica* antiserum. The fractions obtained after three precipitations in 35% SAS at each tem-

perature were practically identical (Table 6).

## DISCUSSION

For most immunological applications, the purpose of serum fractionation is to separate and recover the gamma- or immunoglobulins. Except for the column fractionation methods and even as a preparative step for these techniques, the most widely used method of serum fractionation is precipitation in 50% SAS. Three precipitations are usually recommended to free the material of hemoglobin, but frequently the adequacy of the fractionation in terms of the protein composition of the final product is not determined. When rabbit, sheep, horse, and goat sera were fractionated by three precipitations in 50% SAS, all of the final fractions (Fig. 3) contained more beta and alpha globulins and more albumin than gamma globulin. By using the optimal concentration of $(NH_4)_2SO_4$ for a given animal species of serum, all of the final fractions (Fig. 8) contained predominantly more gamma globulin than any other protein with very little or no albumin.

More than 80% of the gamma globulins present in the original rabbit, sheep, and goat sera were recovered by the improved procedures. The results with horse serum were not as satisfactory. After the serum was precipitated three times in 30% SAS, the high percentage of beta and alpha globulins was reduced, but only 44% of the gamma globulin was recovered. This fraction was 83% gamma globulin and contained no albumin. Approximately 60% of the horse gamma globulin was recovered after only two precipitations in 30% SAS, and, after that step, the fraction contained 74% gamma globulin with only 1% albumin. The recovery of a higher percentage of gamma globulin after two 30% SAS precipitations makes the second precipitate as desirable as the third in 30% SAS, unless the elimination of all albumin is a major objective.

Using 35% SAS, we selectively precipitated rabbit gamma globulin and recovered a high percentage of it regardless of the percentage in the original antiserum. The gamma globulin concentrations (5 to 21%) in the eight rabbit antisera reported in Table 2 were not correlated with the percentage of gamma globulin obtained in the product after fractionation. The gamma globulin recovered from these antisera ranged from 84 to 91% of the original concentration.

These serum fractionation procedures were reliable under variable reaction conditions. At the values tested, the composition of the fractions was not altered by the *p*H of the ammonium sulfate solutions, probably because the buffering capacity of serum kept the *p*H of the mixture within a very narrow range. However,

TABLE 4. *Composition (%) by CASE[a] of rabbit serum fractions obtained with three concentrations of $(NH_4)_2SO_4$ at two different pH levels*

| Proteins in third ppt[b] | 45% SAS[c] | | 40% SAS | | 35% SAS | |
|---|---|---|---|---|---|---|
| | *p*H 5.8 | *p*H 7.2 | *p*H 5.8 | *p*H 7.2 | *p*H 5.8 | *p*H 7.2 |
| Gamma | 35 | 35 | 51 | 46 | 55 | 56 |
| Beta-alpha | 63 | 63 | 48 | 52 | 45 | 44 |
| Albumin | 2 | 2 | 1 | 2 | 0 | 0 |

[a] Cellulose acetate strip electrophoresis.
[b] Precipitate.
[c] Saturated $(NH_4)_2SO_4$.

TABLE 6. *Composition (%) by CASE[a] of the third $(NH_4)_2SO_4$ precipitate of rabbit serum obtained at three temperature levels*

| Proteins | 35% SAS[b] | | |
|---|---|---|---|
| | 20 C | 25 C | 30 C |
| Gamma | 59 | 56 | 57 |
| Beta-alpha | 41 | 44 | 43 |
| Albumin | 0 | 0 | 0 |

[a] Cellulose acetate strip electrophoresis.
[b] Saturated $(NH_4)_2SO_4$.

TABLE 5. *Composition (%) by CASE[a] of rabbit serum fractions obtained from diluted and undiluted serum*

| Proteins | 40% SAS[b], 1st ppt[c] | | | 40%, 2nd ppt | | | 40%, 3rd ppt | | |
|---|---|---|---|---|---|---|---|---|---|
| | Und[d] | 1:2 | 1:10 | Und | 1:2 | 1:10 | Und | 1:2 | 1:10 |
| Gamma | 34 | 36 | 43 | 44 | 48 | 50 | 51 | 49.8 | 54 |
| Beta-alpha | 45 | 44 | 43 | 52 | 50 | 48 | 48 | 49.8 | 44.5 |
| Albumin | 21 | 20 | 14 | 4 | 2 | 2 | 1 | 0.4 | 1.5 |

[a] Cellulose acetate strip electrophoresis.
[b] Saturated $(NH_4)_2SO_4$.
[c] Precipitate.
[d] Undiluted.

diluted serum has less buffering ability, and, if it is fractionated with ammonium sulfate that has not been neutralized, careful attention should be given to *p*H to avoid acid reaction conditions. No differences were noted in the composition of fractions obtained by precipitation of rabbit antiserum with 35% SAS at 20, 25, and 30 C; this finding indicates that variations in temperature within these limits had no effect. Because the molarity of SAS varies with temperature, no attempt has been made to express concentration in molar equivalents, but the SAS used at approximately 25 C was approximately 4.09 M. The data indicated that the composition of fractions prepared from undiluted serum was approximately the same as the composition of fractions prepared from serum diluted either 1:2 or 1:10. The reliability of these procedures depends upon proper technique. Ammonium sulfate solutions must be slowly added to serum while it is gently stirred. When sulfate solutions are poured into sera and the reaction mixtures are shaken or stirred vigorously, the final fractions contain considerably more albumin and beta and alpha globulins than do properly prepared fractions.

Refractionation of very crude 50% SAS globulins with a single precipitation in the optimal SAS concentration for the particular animal species greatly improved the composition of the fractions. Most of the albumin and a large portion of the beta and alpha globulins were effectively removed.

Crude FITC conjugates were also successfully refractionated with optimal SAS concentrations. Only very small amounts of the labeled gamma globulins were lost, and the specific titers did not change. Approximately one-third of the total labeled proteins were removed, and the FITC concentrations were reduced more than 50%. Because of the direct relationship between FITC concentration and nonspecific staining (4), the improved conjugates should exhibit less nonspecific staining than before. The large amount of FITC removed by reducing the percentage of labeled beta and alpha globulins and eliminating the labeled albumin reflects the high avidity of these proteins for FITC (8); it also illustrates the need for employing optimum procedures for fractionation before the antibody is labeled with a fluor.

## ACKNOWLEDGMENT

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